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REMARKS

In this Amendment, Applicant has cancelled Claims 55, 58, 59, 67 – 70, 72, 75, 76, 78, 81 – 84 and 94 – 98, without prejudice or disclaimer, and has amended Claims 52, 56, 57, 71, 73, 77, 79 and 80. Claims 52, 56, 57, 71, 73, 77, 79 and 80 have been amended to further specify the invention and overcome the rejections. Applicant recognizes that the previously presented Claim 59 was only objected as to form. The presently amended Claim 56 includes the same subject matter as the previously presented Claim 59. Therefore, Claim 56 is allowable. In addition, it is respectfully submitted that no new matter has been introduced by the amended claims. All claims are now present for examination in view of the accompanying remarks.

The specification has been amended to correct a clerical error. The amendment to the specification is entirely editorial in nature. It is respectfully submitted that no new matter has been introduced by the amended specification.

REJECTIONS UNDER 35 U.S.C. § 112, SECOND PARAGRAPH:

Claims 52, 58, 61, 64, 67 – 80 and 93 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

Applicant traverses the rejection. It is respectfully submitted that in view of presently claimed invention, the rejection has been overcome. In particular, Claims 58, 67 – 70, 72, 75, 76 and 78 have been cancelled without prejudice or disclaimer. It is respectfully submitted that the Examiner has misunderstood the Claim 52 as previously presented. The "antagonists" at the end of the previously presented Claim 52 only modifies CRF, not other compounds. In the present amendment, Claim 52 has been amended to specify "a composition comprising at least one amino acid selected from the group consisting of valine, leucine, and isoleucine; at least one

antistress agent selected from metyrapone, alphahelical corticotropin releasing hormone, diazepam, allopregnanolone, dextromethorpon, zimelidine, and paroxetine; and a further antistress agent is selected from the group consisting of mifepristone, proglumide, and astressin." Therefore, the compounds included in Claim 52 are definite. By the dependency on Claim 52, Claims 61, 64, 71, 73, 74, 77, 80 and 93 also include the above compounds.

In addition, Claim 71 has been amended to specify "the composition according to claim 52 which further comprises a pyrrolopyrimidine or N-methylpyrrolidone." The support can be found in the specification on page 14 – 15, especially lines 1 – 4 on page 15. The two references incorporated by reference in the specification are enclosed herein for Examiner's reference. They clearly support the compounds defined in Claim 71. Therefore, Claim 71 is definite.

Claim 73 as amended further includes the compound defined in previously presented Claim 76. It is respectfully submitted that "antibiotics" are well defined groups of compounds. Unlimited antibiotics are not available. Mannan oligosaccharides are also well defined group. BioMos™ is provided as an example of such a mannan oligosaccharide product. As indicated in the specification (page 8), the term "performance enhancer" is used to cover those antibiotics and oligosaccharides that are prophylactic for therapeutic agent against disease, particularly in the pig and poultry industry. These are readily identifiable to a person of ordinary skill in the relevant art to prepare animal feeds. Therefore, the scope of Claim 73 as presently defined is clear and definite.

Claim 77 has been amended to correct a clerical error in the spelling of "bolus." In addition, it clearly defines that the composition is encapsulated in a bolus or a time release capsule.

Claim 80 has been amended to specify "the composition according to claim 52 which further comprises a pharmaceutically or veterinarily acceptable diluent, excipient, carrier or solubiliser."

Therefore, the rejection to Claims 52, 58, 61, 64, 67 – 80 and 93 under 35 U.S.C. § 112, second paragraph has been overcome. Accordingly, withdrawal of the rejection under 35 U.S.C. § 112 is respectfully requested.

REJECTIONS UNDER 35 U.S.C. § 112, FIRST PARAGRAPH:

Claims 52, 58, 61, 64, 67 – 80 and 93 have been rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement.

Applicant traverses the rejection. It is respectfully submitted that in view of presently claimed invention, the rejection has been overcome. In particular, Claims 58, 67 – 70, 72, 75, 76 and 78 have been cancelled without prejudice or disclaimer. As stated above, the "antagonists" at the end of the previously presented Claim 52 only modifies CRF, not other compounds. In the present amendment, Claim 52 has been amended to specify "a composition comprising at least one amino acid selected from the group consisting of valine, leucine, and isoleucine; at least one antistress agent selected from metyrapone, alphahelical corticotropin releasing hormone, diazepam, allopregnanolone, dextromethorpon, zimelidine, and paroxetine; and a further antistress agent is selected from the group consisting of mifepristone, proglumide, and astressin." Therefore, the compounds included in Claim 52 are definite and sufficiently supported by the specification. By the dependency on Claim 52, Claims 61, 64, 71, 73, 74, 77, 80 and 93 also include the above compounds. A person of ordinary skill in the relevant art will clearly understand the embodiments of the present invention as defined in Claims 52, 61, 64, 73, 74, 77, 80 and 93.

Therefore, the rejection to Claims 52, 58, 61, 64, 67 – 80 and 93 under 35 U.S.C. § 112, first paragraph has been overcome. Accordingly, withdrawal of the rejection under 35 U.S.C. § 112 is respectfully requested.

REJECTION UNDER 35 U.S.C. § 102(b)

Claims 52, 58, 61, 64, 67 – 80 and 93 have been rejected under 35 U.S.C. § 102(b), as allegedly being anticipated by the Schaefer et al (US 5,728,675), hereinafter Schaefer.

Applicant traverses the rejection. At first, Claims 58, 67 – 70, 72, 75, 76 and 78 have been cancelled without prejudice or disclaimer. The rejection therefore is moot. Secondly, as stated above, the "antagonists" at the end of the previously presented Claim 52 only modifies CRF, not other compounds. In the present amendment, Claim 52 has been amended to specify "a composition comprising at least one amino acid selected from the group consisting of valine, leucine, and isoleucine; at least one antistress agent selected from metyrapone, alphahelical corticotropin releasing hormone, diazepam, allopregnanolone, dextromethorpon, zimelidine, and paroxetine; and a further antistress agent is selected from the group consisting of mifepristone, proglumide, and astressin." Obviously, Schaefer does not disclose the specific components as required in Claim 52. Due to their dependency on Claim 52, Claim 61, 64, 71, 73, 74, 77, 80 and 93 are also distinguished from the disclosures in Schaefer.

Therefore, the rejection under 35 U.S.C. § 102(b) has been overcome. Accordingly, withdrawal of the rejection under 35 U.S.C. § 102 (b) is respectfully requested.

REJECTIONS UNDER 35 U.S.C. § 103:

Claims 52, 55 – 57, 61, 67, 68, 80 and 93 have been rejected under 35 U.S.C. § 103, as allegedly being obvious and unpatentable over Blackburn (WO 82/0041) in view of Walser (WO 95/30418) or Ito et al (US 5,937,790) and Daley et al '93. Applicant traverses the rejection.

Blackburn discloses a therapeutic amino acid preparation for the treatment of stress and injury. Specifically, the pharmaceutical preparation contains 70% to 100% of the branched chain amino acids valine, leucine and isoleucine. The amino acids are said to promote protein synthesis in a stressed body. The compositions are formulated with a variety of diluents,

excipients, carriers and infusion solutions and pH adjusters. However, there is no suggestion or motivation that these amino acids should be used together with anti-stress agents, especially at least one antistress agent selected from metyrapone, alphahelical corticotropin releasing hormone, diazepam, allopregnanolone, dextromethorpon, zimelidine, and paroxetine; and a further antistress agent is selected from the group consisting of mifepristone, proglumide, and astressin.as included in the embodiments of the present invention as claimed.

Walser discloses a method for treating HIV by administering an agent that suppresses endogenuous production of glucocorticoids together with a replacement glucocorticoid. The agent includes metyrapone and mifepristone. However, stress is not specifically mentioned as being treated. There is no suggestion or motivation to use the method disclosed in Walser to treat stress. Examiner may assume that HIV treatment itself causing stress. However, the present invention specifically defined that the stresses being treated are stresses from injury, trauma, surgery, hunger, thirst, fatigue, thermal extreme stress (page 17, lines 5 - 6). There is no indication or appreciation of synergistic interaction to combine the amino acids in Blackburn with the therapeutic method in Walser to achieve the present invention as presently claimed.

Ito discloses anti-stress agents for animals and a method for reducing stress in animals. Specifically, Ito teaches L-ascorbic acid-2-phosphoric acid, a salt thereof and an L-ascorbic acid-2-glucoside as active ingredients. Although Vitamin C combination with oligosaccharides are allegedly shown by relying on 2-O-.alpha.-D-glucopyranosyl-L-ascorbate (col. 6, line 41), there is no teaching or suggestion that another anti-stress agent can also be used and that it will enhance the anti-stress effect in combination with the amino acid to achieve the present invention as claimed

Finally, Daley discloses the use of metyrapone to treat wounds in a cat with pituitary dependant hyperadrenocorticism. Similar to Walser, Daley does not indicate that metyrapone is used as an anti-stress agent or its use with particular amino acids as included by the embodiments of the present invention.

Therefore, neither Blackburn, Walser, Ito nor Daley have suggestion or incentive to combine these references together to achieve the present invention. Even if combined, they do not teach or suggest the invention as presently claimed. Accordingly, Applicant respectfully requests that the rejection under 35 U.S.C. §103 be withdrawn.

Having overcome all outstanding grounds of rejection, the application is now in condition for allowance, and prompt action toward that end is respectfully solicited.

Respectfully submitted,

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Journal of Neuroscience Research 47:650–654 (1997)

Neuroprotective Effects of the Novel Brain-Penetrating Pyrrolopyrimidine Antioxidants U-101033E and U-104067F Against Post-Ischemic Degeneration of Nigrostriatal Neurons

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A 10-min period of bilateral carotid occlusion (BCO)-induced forebrain ischemia in gerbils triggers a delayed retrograde degeneration of 35–40% of dopaminergic nigrostriatal (NS) neurons. The mechanism of the NS degeneration is believed to involve oxygen radical formation secondary to a postischemic increase in dopamine turnover (monoamine oxidase, MAO). If the oxygen radical increase is sufficiently severe, lipid peroxidative injury to the striatal NS terminals is followed by retrograde degeneration of the NS cell bodies. In the present study, we examined whether the novel brain-penetrating lipid antioxidant pyrrolopyrimidine, U-101033E, and its aromatized analog, U-104067F, could attenuate dopaminergic neurodegeneration in this model. Male Mongolian gerbils were dosed with U-101033E (1.5, 5, or 15 mg/kg, by mouth, twice daily) or U-104067F (5 or 15 mg/kg, by mouth, twice daily) for 27 days beginning on the day of the 10-min ischemic insult. Preservation of NS neurons was assessed by tyrosine hydroxylase immunohistochemistry at 28 days. In vehicle (40% hydroxypropyl-β-cyclodextrin)-treated animals, there was a 42% loss of NS neurons. In contrast, gerbils that received 5 or 15 mg/kg U-101033E twice daily had only a 23% or 28% loss of NS neurons, respectively ($P < 0.002$ vs. vehicle). U-104067F showed little effect at sparing neurons at the 10 mg/kg dose, but did significantly attenuate neuronal loss to only 20% at the 30 mg/kg dose ($P < 0.01$ vs. vehicle). The results show that both the pyrrolopyrimidines (U-101033E and U-104067F) significantly attenuate the postischemic loss of NS dopaminergic neurons and further support the involvement of a dopamine metabolism-derived, oxygen radical-induced lipid peroxidative mechanism. J. Neurosci. Res. 47:650–654, 1997. © 1997 Wiley-Liss, Inc.

Key words: pyrrolopyrimidine; ischemia; nigrostriatal; degeneration; neuroprotection

INTRODUCTION

Parkinson's disease is a major neurodegenerative disorder in which there is a progressive loss of nigrostriatal (NS) dopaminergic neurons (Ishii et al., 1993). It has become increasingly clear that oxygen radical-induced, iron-catalyzed lipid peroxidation probably plays a role in the NS degeneration (Cohen, 1986; Jenner et al., 1992; Ishii et al., 1993; Youdim et al., 1993). The progressive death of NS dopaminergic neurons leads to a dramatic decrease in striatal dopamine and the classic Parkinsonian symptomatology including tremor, rigidity, and bradykinesia (later akinesia). The surviving dopamine neurons attempt to compensate by increasing their release of neurotransmitter. However, the increased turnover of dopamine, which includes increased metabolism by monoamine oxidase (MAO), may actually serve to increase the oxidative stress that the NS neurons may already be undergoing. This is due to the fact that MAO activity causes the generation of hydrogen peroxide that, in turn, through Fenton chemistry gives rise to hydroxyl radicals ($Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + {}^{\cdot}OH + OH$) (Cohen, 1986; Spina and Cohen, 1989; Youdim et al., 1993). The relationship between MAO activity-related oxidative stress and NS degeneration has also recently been demonstrated in the context of the gerbil forebrain ischemia model. Brief episodes of near complete forebrain ischemia have been shown to trigger a progressive degeneration of NS neurons over a 28-day period (Shuaib et al., 1992; Saji and Volpe, 1993; Hall et al., 1994; Saji et al., 1994; Andrus et al., 1995). The onset of degeneration is preceded by an initial postreperfusion increase in striatal dopamine turnover and ·OH degeneration (Hall et al.,

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1994; Andrus et al., 1995). A likely mechanistic candidate for a linkage between the postischemic dopamine turnover and associated oxidative stress and the degeneration of the NS neurons is free radical-induced lipid peroxidation (Braughler and Hall, 1989; Hall and Braughler, 1989). If lipid peroxidation is indeed the mechanism for NS degeneration after ischemia, then lipid antioxidant compounds would be expected to attenuate the progressive postischemic, Parkinson-like degeneration of NS neurons.

In the present study, we have examined the ability of two novel, brain-penetrating pyrrolopyrimidine lipid peroxidation inhibitors, U-101033E and U-104067F, to limit the progressive loss of NS neurons in gerbils measured at 28 days after a 10-min episode of near-complete forebrain ischemia. In other studies, these compounds have been shown to effectively attenuate the loss of the highly vulnerable hippocampal CA_1 neurons in gerbils following a 5-min ischemic insult (Hall et al., 1995).

MATERIALS AND METHODS

Gerbil Forebrain Ischemia Procedure

All experiments received prior approval by the Corporate Animal Welfare Committee of Upjohn Laboratories to ensure that they were performed in strict compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Male Mongolian gerbils, obtained from Tumblebrook Farms (West Brookfield, MA) and weighing 45–55 g, were anesthetized with methoxyflurane. A 1- to 2-cm midline throat incision provided access to both carotid arteries, which were clamped with microaneurysm clamps. The animals were placed in a warming box with an ambient temperature maintained at 37°C for the duration of the ischemic insult. After 10 min of near-complete ischemia, the clamps were removed and reperfusion allowed for 28 days.

Pyrrolopyrimidine Dosing

Animals were treated with vehicle (40% aqueous hydroxypropyl-β-cyclodextrin; Encapsin HPB, American Maize-Products, Hammond, IN) or dosed with U-101033E (by mouth, twice a day) at 1.5, 5, or 15 mg/kg twice on the day of ischemia and then twice daily thereafter for the 4-wk reperfusion phase. One group of gerbils received U-101033E at 30 mg/kg once daily. In a separate experiment, animals were similarly dosed with either vehicle or with U-104067F (by mouth, twice a day) at 5 or 15 mg/kg. In both experiments, sham-occluded gerbils underwent the same anesthesia and surgical procedures without the ischemic insult. Doses were selected based on previous studies showing the ability of the two compounds to prevent ischemic damage to hippocampal CA_1 neurons in gerbils (Hall et al., 1995) or to retard retrograde degeneration of axotomized facial motor neurons in neonatal rats (Smith et al., 1996).

Tyrosine Hydroxylase Immunohistochemistry

After 28 days, the animals were deeply reanesthetized with methoxyflurane and perfused intracardially with oxygenated Krebs-Ringer bicarbonate (pH 7.2) until the effluent was cleared of blood (2 min), followed by perfusion with cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 9 min (approximately 200 ml). The brains were removed, blocked, and postfixed for 4 hr at 4°C. The brain tissues were equilibrated in increasing concentrations of sucrose (10%, 20%, 30%) in phosphate-buffered saline (PBS), pH 7.4, over a 3-day period. After complete equilibration in 30% sucrose, the brains were frozen in liquid nitrogen vapor for 10 min and stored at −70°C until processing. The brains were later sectioned at 50 μm on a Leitz (Nuhsbaum, Inc., McHenry, IL) sledge microtome into 0.01 M PBS at 4°C (pH 7.4). Serial sections through the substantia nigra were processed as free-floating sections for tyrosine hydroxylase (TH) immunohistochemistry.

The number of TH-positive neurons was manually counted in a strip of three reticules, each 620 × 620 μm, at 20 × along the substantia nigra (zona compacta and zona reticula). The raw numbers were converted to the number of TH-positive neurons/mm². The numbers of neurons/mm² for the two sides were averaged for each animal. Neurons were counted in three regions (planes) through the substantia nigra (region A = 1.61 mm; region B = 1.76 mm; region C = 1.95 mm). All evaluations were blinded as to which slides were from vehicle- or drug-treated animals. Statistical evaluation was performed using a Student's *t*-test with Bonferoni correction for multiple comparisons.

RESULTS

Figure 1 shows the multiregional dose-response relationship for the neuroprotective effect of U-101033E on the retrograde degeneration of the TH-positive (dopamine) neurons in the substantia nigra 28 days after a 10-min episode of near-complete forebrain ischemia in the gerbil. There was a significant 42% loss ($P < 0.001$) of TH-positive NS neurons in the substantia nigra in vehicle-treated animals compared with sham (nonoccluded) animals. Other work has demonstrated that the loss of TH-stained neurons represents a real loss of NS neurons because cresyl violet staining shows an equivalent loss at 28 days after ischemia (data not shown).

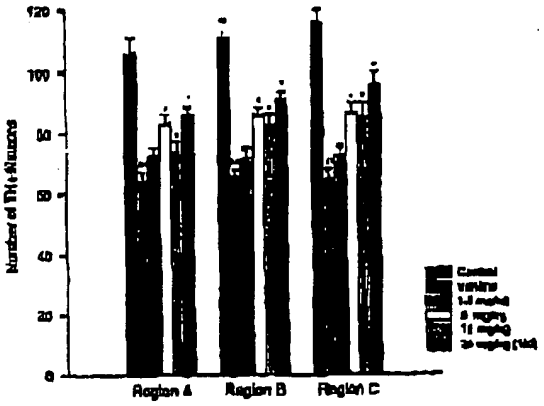

Fig. 1. Dose-response (p.o., BID) and multi-regional analysis of U-101033E's effect on the delayed degeneration of dopaminergic tyrosine hydroxylase (TH)-positive nigral neurons at 28 days after a 10-min episode of near complete forebrain ischemia in male gerbils. Each bar represents mean ± SEM for 10–14 animals. $^{**}P < 0.001$ versus sham; $^{*}P < 0.01$ versus vehicle.

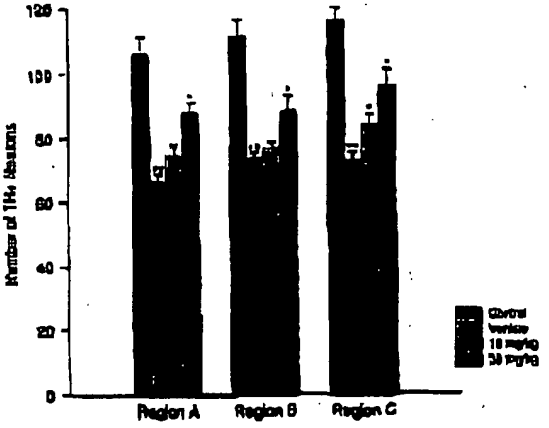

Fig. 2. Dose-response and multi-regional analysis of U-104067F's effect on the delayed loss of dopaminergic tyrosine hydroxylase-positive nigral neurons at 28 days after a 10-min episode of near complete forebrain ischemia in male gerbils. Each bar represents mean ± SEM for 11–12 animals. $^{**}P < 0.001$ versus sham; $^{*}P < 0.01$ versus vehicle.

U-101033E produced slight protection of the NS neurons at the 1.5 mg/kg dose (by mouth, twice daily) and significant sparing at the 5 mg/kg dose (by mouth, twice daily; $P < 0.001$) and at the 15 mg/kg dose (by mouth, $P < 0.002$) in each of the three regions of the substantia nigra that were examined. The group of animals receiving U-101033E at 30 mg/kg once daily also showed significant protection ($P < 0.001$).

Figure 2 shows the multiregional dose-response relationship for the neuroprotective effect of U-104067F in the same model. Again, there was a significant loss of TH-positive neurons in the substantia nigra in vehicle-treated animals compared with sham animals. U-104067F demonstrated significant sparing ($P < 0.05$) of NS neurons at the 10 mg/kg dose, but only in region C of the substantia nigra. However, at 30 mg/kg, the compound significantly ($P < 0.01$) protected in each of the three regions that were examined. Typical examples of the postischemic loss of NS neurons and the partial protective effects of U-104067F are shown in Figure 3.

DISCUSSION

Our results show that a single 10-min episode of forebrain ischemia in gerbils triggers a progressive degeneration of dopaminergic NS neurons that offers a model of Parkinson's Disease that is potentially modifiable by drug treatment. The current model and the demonstrated neuronal damage are consistent with that seen by others in similar gerbil forebrain ischemic insults (Shuaib et al., 1992; Saji and Volpe, 1993; Saji et al., 1994). In the gerbil forebrain ischemia model and similar rodent forebrain ischemia paradigms, there is significant postischemic increase in striatal dopamine turnover (Weinberger and Cohen, 1983; Globus et al., 1987a,b; Ishii et al., 1993; Simonson et al., 1993; Hall et al., 1994) that is coincident with an increase in mitochondrial MAO activity (Ishii et al., 1993), hydroxyl radical levels (Delbarre et al., 1993; Hall et al., 1993, 1994; Simonson et al., 1993), and lipid peroxidation products (Ishii et al., 1993). This mechanistic cascade is consistent with the concept that the initial ischemia-triggered increase in dopamine release and metabolism-derived oxygen radicals initiates a lipid peroxidative injury to the NS terminals in the striatum that, if sufficiently severe, results in a delayed retrograde degeneration of the NS cell bodies in the substantia nigra.

If lipid peroxidation is the mechanism for NS degeneration after ischemia, then lipid antioxidant compounds would be expected to attenuate the progressive postischemic, Parkinson-like degeneration of NS neurons. Consistent with this mechanistic view, we have demonstrated that U-101033E and U-104067F—two members of a novel group of brain-penetrable, potent inhibitors of iron-dependent lipid peroxidation in the neural tissue that operate by electron-donating and radical trapping mechanisms (Hall et al., 1995)—effectively attenuate the postischemic loss of NS neurons. Although neither compound is completely effective, both reduced

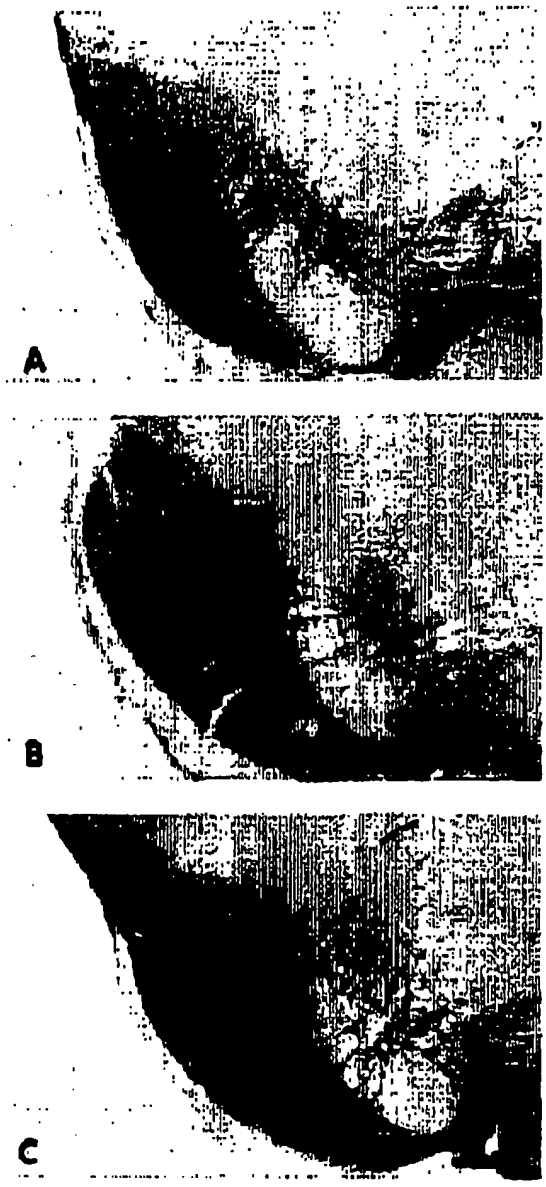

Fig. 3. A: Comparison of the tyrosine hydroxylase-positive nigral neurons present in normal, non-occluded animals. B: The loss of neurons in vehicle-treated animals at 28 days after a 10-min episode of near-complete forebrain ischemia in male gerbils. C: The protective effect of the pyrrolopyrimidine U-104067F on the retrograde degeneration of the tyrosine hydroxylase-positive neurons in gerbils that had undergone the same surgical procedures. Bar = 1 mm.

the degeneration by at least half. Because the delayed and progressive postischemic loss of the NS neurons is reminiscent of NS degeneration in Parkinson's disease, chronic treatment with a pyrrolopyrimidine may serve to slow the progression of that disorder, which has been increasingly attributed to an oxygen radical-induced, iron-dependent, lipid peroxidative process (Cohen, 1986; Jenner et al., 1992; Ishii et al., 1993; Youdim et al., 1993).

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Neuroprotective Efficacy of Microvascularly-Localized Versus Brain-Penetrating Antioxidants

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Summary

The 21-aminosteroid (lazaroid) tirilazad mesylate has been demonstrated to be a potent inhibitor of lipid peroxidation and to reduce traumatic and ischemic damage in a number of experimental models. Currently, tirilazad is being actively investigated in Phase III clinical trials in head and spinal cord injury, ischemic stroke and subarachnoid hemorrhage. This compound acts in large part to protect the microvascular endothelium and consequently to maintain normal blood-brain barrier (BBB) permeability and cerebral blood flow autoregulatory mechanisms. However, due to its limited penetration into brain parenchyma, tirilazad has generally failed to affect delayed neuronal damage to the selectively vulnerable hippocampal CA1 and striatal regions. Recently, we have discovered a new group of antioxidant compounds, the pyrrolopyrimidines, which possess significantly improved ability to penetrate the BBB and gain direct access to neural tissue. Several compounds in the series, such as U-101033E, have demonstrated greater ability to protect the CA1 region in the gerbil transient forebrain ischemia model with a post-ischemic therapeutic window of at least four hours. In addition, U-101033E has been found to reduce infarct size in the mouse permanent middle cerebral artery occlusion model in contrast to tirilazad which is minimally effective. These results suggest that antioxidant compounds with improved brain parenchymal penetration are better able to limit certain types of ischemic brain damage compared to those which are localized in the cerebral microvasculature. On the other hand, microvascularly-localized agents like tirilazad appear to have better ability to limit BBB damage.

Keywords: Antioxidant; tirilazad; pyrrolopyrimidine; ischemia.

Introduction

There is now a significant amount of information that supports a role of oxygen radical-induced lipid peroxidation (LP) in the pathophysiology of acute central nervous system injury and ischemia [4,7,20]. The 21-aminosteroid (lazaroid) tirilazad mesylate has been demonstrated to be a potent inhibitor of LP that acts by a combination of chemical radical scavenging and membrane stabilization mechanisms. It has been shown to reduce traumatic and ischemic damage in a number of experimental models, and a correlation has been demonstrated in several instances between attenuation of oxygen radical levels and/or lipid peroxidation and the neuroprotective effect (see review by Hall *et al.*, [9]). Currently, tirilazad is being actively investigated in Phase III clinical trials in head and spinal cord injury, ischemic stroke and subarachnoid hemorrhage (SAH). Results from a multi-national European/Australian/New Zealand trial in SAH have demonstrated a highly significant reduction in 3 month mortality and improvement in the incidence of "Good" recovery (Glasgow Outcome Scale) in patients treated with tirilazad [11].

Tirilazad appears to act in large part on the microvascular endothelium [1,16] and consequently has been shown to protect the blood-brain barrier (BBB), to maintain cerebral or spinal cord blood flow autoregulatory mechanisms and/or to reduce delayed vasospasm in multiple models [9]. Therefore, its' ability to protect neural tissue from traumatic or ischemic insult in many models may be largely indirect. Indeed, tirilazad, most likely due to its limited penetration into brain parenchyma [19], has generally failed to affect delayed neuronal damage in the selectively vulnerable hippocampal CA1 and striatal regions [2,5,12,21], although it has some effect to protect cortical neurons [12,21]. Moreover, in models of permanent focal ischemia where microvascular effects may be less important than in temporary ischemia paradigms, the

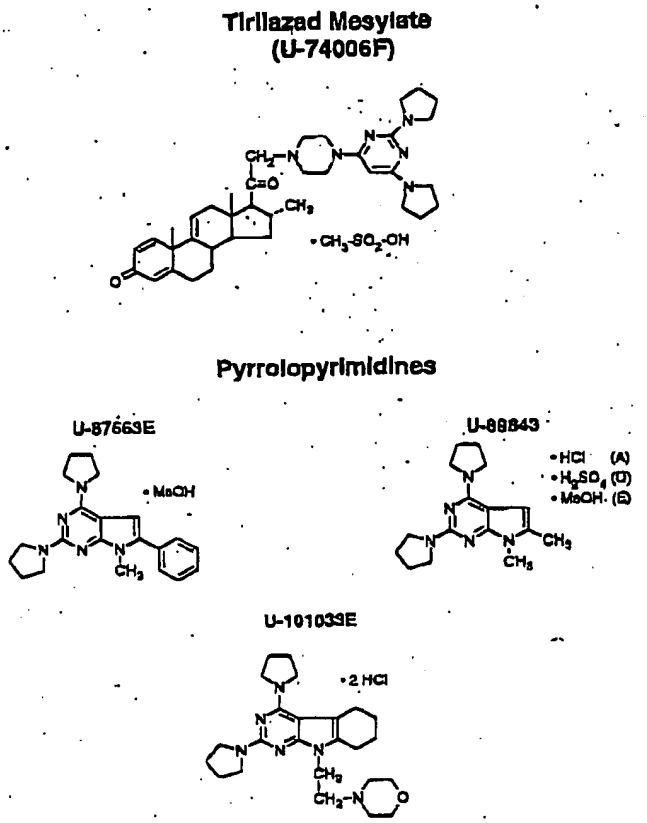

Fig. 1. Chemical structures of the 21-aminosteroid or lazaroid tirilazad mesylate (U-74006F) and selected pyrrolopyrimidines

compound's ability to affect infarct size, while demonstrated in some experiments [3,15], has been inconsistent [22].

Thus, we reasoned that lipid peroxidation-inhibiting (antioxidant) compounds with improved brain penetration might possess certain advantages over the microvascularly-localized tirilazad in certain CNS injury situations. Recently, we have discovered a new group of compounds, the pyrrolopyrimidines (Fig. 1), which are equal or better antioxidants than tirilazad, but with significantly improved ability to penetrate the BBB and gain direct access to neural tissue. Several compounds in the series, including U-101033E, have demonstrated greater ability than tirilazad to protect the CA1 region in the gerbil forebrain ischemia model with a post-ischemic therapeutic window of at least four hours. In addition, U-101033E has been found to reduce infarct size in the mouse permanent middle cerebral artery occlusion model in contrast to tirilazad which is inactive. On the other hand, microvascularly-localized agents like tirilazad appear to have better ability to limit BBB damage after experimental SAH.

Structural Comparison of Tirilazad Mesylate and the Pyrrolopyrimidines

Figure 1 displays the chemical structures of tirilazad mesylate and three of the pyrrolopyrimidines, U-87663E (the original prototype), U-89843D and U-101033E*. Tirilazad is a non-glucocorticoid 21-aminosteroid or "lazaroid". The primary chemical antioxidant portion of the molecule is the amino moiety bound to the 21 position of the steroid side chain. Physicochemical studies indicate that the highly lipophilic (i.e. hydrophobic) steroid moiety orients itself within the hydrophobic fatty acid core of the membrane and is largely responsible for the high affinity of the compound for cell (e.g. endothelium) membranes [9,19]. The more hydrophilic amino substitution exists closer to the surface in juxtaposition to the phosphate head groups of the phospholipids. In contrast, the pyrrolopyrimidines lack the highly lipophilic steroid moiety, while bearing some structural resemblance to the bispyrrolidinylpyrimidinyl piperazine 21-amino substitution of tirilazad. The lack of the steroid serves to lessen the high affinity for and retention in lipid bilayers (T. J. Raub and G. A. Sawada, unpublished results).

Comparison of Inhibition of Iron-Dependent Lipid Peroxidative Neuronal Injury

Table 1 shows the IC_{50}s and maximum % protection of cultured fetal mouse spinal neurons from iron (200 μM ferrous ammonium sulfate)-induced lipid peroxidative injury (system described in detail elsewhere, 8) by tirilazad in comparison to the pyrrolopyrimidines. Protection was measured in terms of preservation of amino acid uptake (i.e. uptake of 3H-amino isobutyric acid). As seen, the pyrrolopyrimidines are generally more potent and slightly more efficacious in this *in vitro* model. However, with both types of compounds, a correlation has been demonstrated between preservation of amino acid uptake and attenuation of iron-induced lipid peroxidation (data not shown).

* The suffix letters indicate the salt form of the compound. Different salts were sometimes studied such as A = hydrochloride, D = sulfuric or E = methane sulfonate. Thus, suffix letters may vary below.

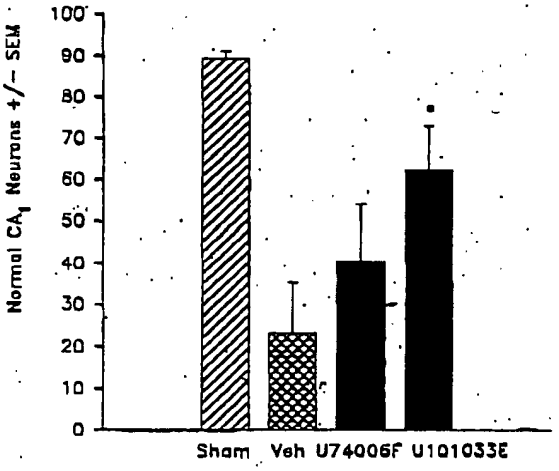

Fig. 3. Comparison of the ability of U-74006F and U-101033E to salvage hippocampal CA1 neurons at 5 days after a 5 min episode of bilateral carotid occlusion in Mongolian gerbils. Values = means ± standard error for 10 animals/group. Gerbils were dosed with 30 mg/kg per os pre-ischemia plus 2 hrs after reperfusion and once daily on days 2,3 and 4. Asterisk indicates $p < 0.05$ vs. the vehicle treated group

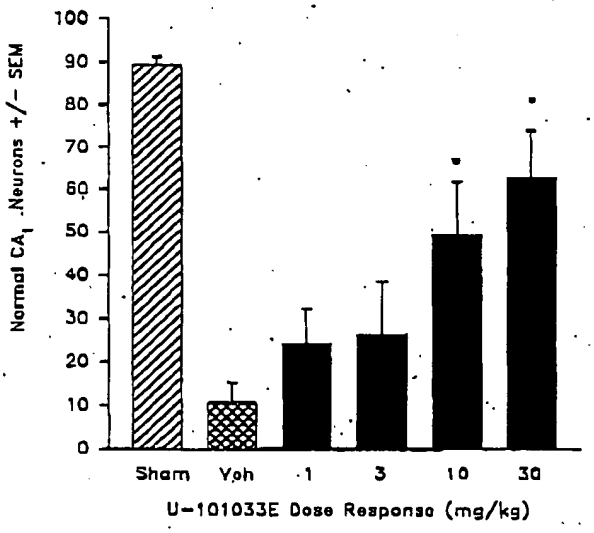

Fig. 4. Dose-response of U-101033E to salvage hippocampal CA1 neurons at 5 days after a 5 min episode of bilateral carotid occlusion in Mongolian gerbils. Values = means ± standard error for 10 animals/group. Gerbils were dosed with each dose level per os pre-ischemia plus 2 hrs after reperfusion and once daily on days 2,3 and 4. Asterisk indicates $p < 0.05$ vs. the vehicle treated group.

forebrain ischemia [2,5,12,15] perhaps due to its limited BBB penetration in the context of models where BBB permeability is minimally compromised. Similarly Fig. 3 shows that preischemic oral treatment with tirilazad (plus additional post-ischemic dosing) has only a small effect on CA1 neuronal preservation in the gerbil 5 min. forebrain ischemia model. In contrast,

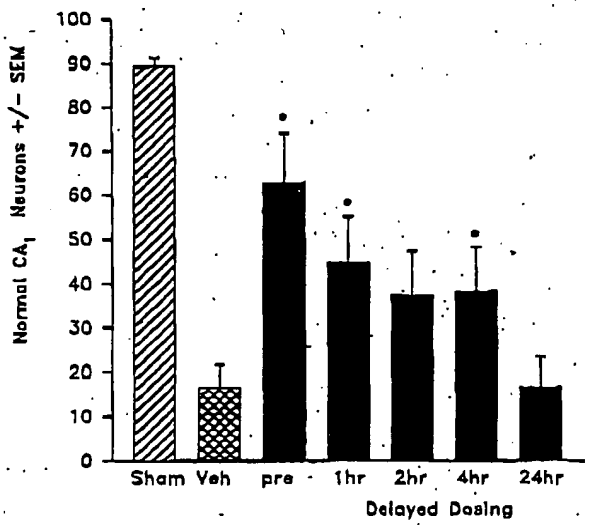

Fig. 5. Therapeutic window of U-101033E to salvage hippocampal CA1 neurons at 5 days after a 5 min episode of bilateral carotid occlusion in Mongolians gerbils. Values = means ± standard error for 10 animals/group. Gerbils were dosed beginning at each time point with 30 mg/kg per os plus 2 hrs later and on the subsequent days. Asterisk indicates $p < 0.05$ vs. the vehicle treated group

oral pretreatment with the more BBB-permeable pyrrolopyrimidine U-101033E produces twice as much CA1 protection compared to tirilazad, reaching a level that is significantly greater than the CA1 preservation observed in vehicle treated animals. Figure 4 shows the dose-response curve for the ability of U-101033E pretreatment to protect the CA1 region. As seen, dose levels of 10 or 30 mg/kg are significantly effective, whereas doses as low as 1 and 3 mg/kg appear to have only some effect.

Therapeutic Window in Transient Forebrain Ischemia

Figure 5 displays the therapeutic window for the efficacy of U-101033E with regard to CA1 protection in the gerbil 5 min. forebrain ischemia model. As can be seen in this graph, the initiation of dosing 30 min. prior to ischemia (plus repeated post-ischemic dosing) is the most effective. However, a delay in dosing to 4 hr. after reperfusion still provides a statistically significant neuroprotective effect.

Comparison of Infarct Reduction in Permanent Focal Ischemia

The neuroprotective efficacy of tirilazad has also been compared to selected pyrrolopyrimidines in the context of focal ischemic models. For instance, both

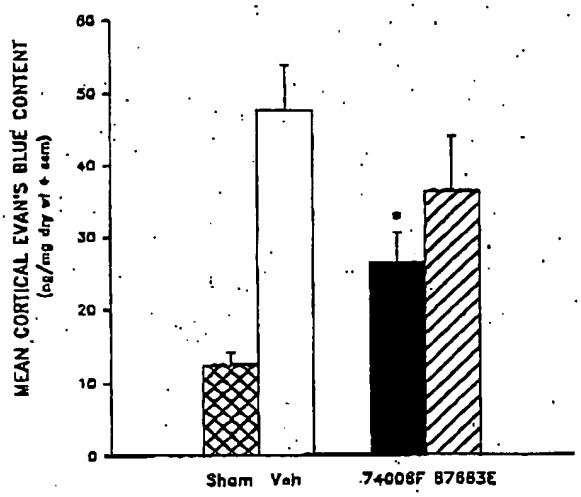

Fig. 7. Comparison of the ability of U-74006F and the pyrrolopyrimidine U-87663E to reduce blood-brain barrier permeability (Evans' blue extravasation) in male Sprague-Dawley rats at 3 hrs after SAH. The animals received a 1 mg/kg i.v. dose of either compound (or an equivalent volume of vehicle) at 15 min before and again at 2 hrs after SAH. Brains were dried and the Evan's blue extracted with formamide and measured fluorometrically. Values = means ± standard error. N = 8–14 animals/group. Asterisk indicates $p < 0.05$ vs. vehicle

tioxidants have been previously described with neuroprotective efficacy in transient forebrain ischemia models in either gerbils or rats including LY-178002 [6], N-tert-butyl-α-phenynitrone (PBN; [17]) and dimethylthiourea (DMTU; [14]). However, much higher doses of all of these compounds appear to be required to achieve neuroprotection as compared to U-101033E which is significantly effective at oral dose levels as low as 10 mg/kg. This suggests that these earlier-described and studied compounds either may not be as brain-penetrable as thought or perhaps they are not as effective at attenuating oxygen radical-induced, iron-catalyzed lipid peroxidation as the pyrrolopyrimidines appear to be. In addition, U-101033E has been shown to have at least a 4 hr post-ischemic therapeutic window. In contrast, PBN's ability to protect CA1 neurons in the identical gerbil forebrain ischemia model is lost by 2 hrs after reperfusion [17]. Nevertheless, further study of U-101033E and other pyrrolopyrimidines is neccessary before an exact assessment of their neuroprotective activity in comparison to the earlier described compounds can be firmly established.

The pyrrolopyrimidines similarly outperform tirilazad, as well as another antioxidant dihydrolipoate [18], with regard to the ability to reduce early infarct growth in the mouse permanent MCA occlusion model. In the face of permanent vascular occlusion, a successful neuroprotective compound must intuitively be able to penetrate the underperfused ischemic penumbra zone in order to be optimally effective in salvaging the still viable, but potentially doomed neural tissue. While tirilazad has been reported to reduce infarct volume in the setting of permanent MCA occlusion in Sprague-Dawley [16] and Fischer [3] rats, it has not been shown to be efficacious in the same model in spontaneously hypertensive rat strain [22]. Likewise, tirilazad appears to be only marginally effective in the mouse permanent MCA model. In contrast, U-87663E, U-89843D and U-101033E all potently decrease infarct size in the same model, most likely due to their greater access to the ischemic brain parenchyma.

Interestingly, the brain-penetrable pyrrolopyrimidines are not superior to tirilazad in the context of either temporary (but prolonged) focal ischemia (gerbil 3 hr unilateral carotid occlusion) or in SAH-induced BBB damage. In the setting of temporary focal ischemia, tirilazad and U-87663E and U-89843A are equally neuroprotective. Two explanations are feasible. First of all, post-reperfusion BBB damage in this model may serve to enhance parenchymal access of tirilazad such that it is able to reach the neurophil. Secondly, BBB damage, and its potential attenuation by the microvascularly-localized tirilazad, may be equally important with parenchymal neuronal injury mechanisms which would be most efficiently countered by the brain-penetrable pyrrolopyrimidines. On the other hand, in the latter setting of SAH-induced BBB damage and the consequent pathological protein permeability, it is apparent that the microvascularly-localized tirilazad has a clear advantage. Thus, within the overall spectrum of traumatic, ischemic and hemorrhagic CNS insults, it would seem that antioxidant compounds that localize in brain microvasculature or that penetrate the brain parenchyma will both have specific, and no doubt complimentary, therapeutic roles to play.

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